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(54) Title: AMPHIPHILIC IMIDAZOLINIUM DERIVATIVES

(57) Abstract

Amphiphiles containing an imidazolinium ring system are provided that are non-toxic to the host mammal. The amphiphiles are used to produce liposomes useful as carriers for delivering macromolecules intracellularly.

AMPHIPHILIC IMIDAZOLINIUM DERIVATIVES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of USSN 08/157,727, filed November 24, 1993, which is a continuation-in-part of USSN 07/991,935, filed December 17, 1992, which disclosures are herein incorporated by reference.

INTRODUCTION

FIELD OF THE INVENTION

This invention relates to nitrogen-containing amphiphiles and their use in the preparation of liposomes and other lipid-containing carriers of pharmaceutical substances, including nucleic acids used in gene therapy.

BACKGROUND OF THE INVENTION

Liposomes are one of a number of lipid-based materials used as biological carriers and have been used effectively as carriers in a number of pharmaceutical and other biological situations, particularly to introduce drugs, radiotherapeutic agents, enzymes, viruses, transcriptional factors and other cellular vectors into a variety of cultured cell lines and animals. Successful clinical trials have examined the effectiveness of liposome-mediated drug delivery for targeting liposome-entrapped drugs to specific tissues and specific cell types. See, for example, U.S. patent No. 5,264,618, which describes a number of techniques for using lipid carriers, including the preparation of liposomes and pharmaceutical

use of naked DNA expression vectors (Nabel et al. (1990), supra; Wolff et al. (1990) Science, 247:1465-1468). Direct injection of transgenic material into tissue produced only localized expression (Rosenfeld (1992) supra); Rosenfeld et al. (1991) supra). Brigham et al. (1989) supra; Nabel (1990) supra; and Hazinski et al. (1991) supra). The Brigham et al. group (Am. J. Med. Sci. (1989) 298:278-281 and Clinical Research (1991) 39 (abstract) have reported *in vivo* transfection restricted to lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. See also Stribling et al. Proc. Natl. Acad. Sci. (USA) 89:11277-11281 (1992) which reports the use of liposomes as carriers for aerosol delivery of transgenes to the lungs of mice and Yoshimura et al. Nucleic Acids Research (1992) 20:3233-3240.

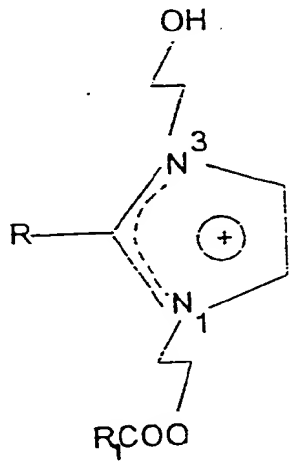
Cationic lipid carriers have been shown to mediate intracellular delivery of plasmid DNA (Felgner, et al., Proc. Natl. Acad. Sci. USA (1987) 84:7413-7416); mRNA (Malone, et al., Proc. Natl. Acad. Sci. USA (1989) 86:6077-6081); and purified transcription factors (Debs, et al., J. Biol. Chem. (1990) 265:10189-10192), in functional form.

SUMMARY OF THE INVENTION

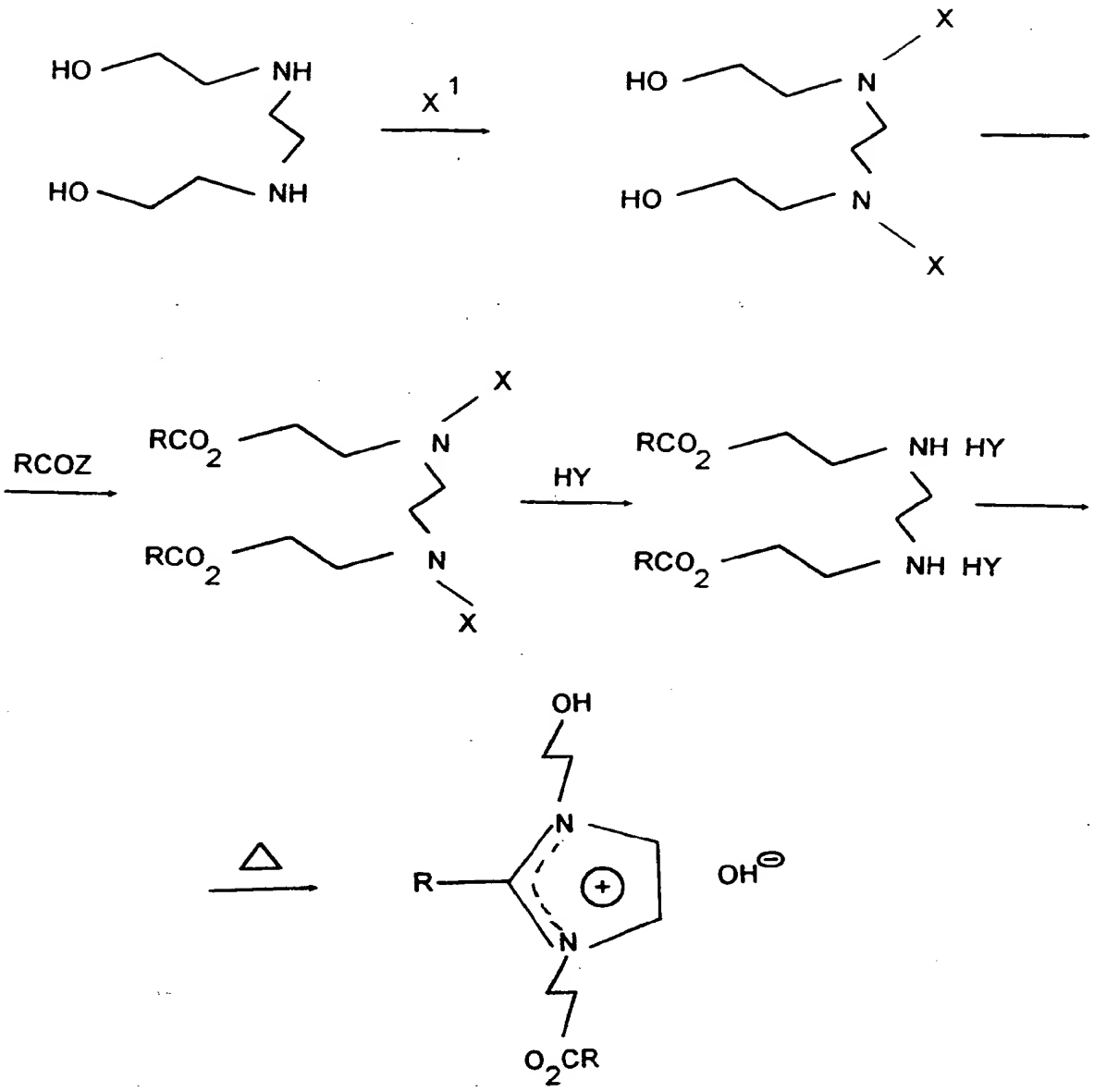
Biodegradable, novel, amphiphilic imidazolinium derivatives are provided as are the methods of their use. The cationic amphiphiles are capable of forming complexes with nucleic acids, and other biological compounds and the nucleic acid complexes are capable of transforming mammalian cells. The amphiphiles of the invention yield non-toxic degradation products when subjected to endogenous enzymatic processes.

example, enzymatic cleavage products, which are nontoxic to a host organism or which are endogenous to a host organism. Generally, both the original lipids and their degradation products are nontoxic to a host organism.

The invention particularly relates to nitrogen-containing amphiphilic cations having the formula:



wherein each of R and R_1 independently is a straight-chain, aliphatic hydrocarbyl group of 11 to 29 carbon atoms inclusive. Preferred are those cations wherein each of R and R_1 independently has from 13 to 23 carbon atoms inclusive. The R and R_1 groups are saturated or are unsaturated having one or more ethylenically unsaturated linkages and are suitably the same or are different from each other. Illustrative R_1 groups together with the -CO- group to which it is attached (i.e., R_1-CO-) include lauroyl, myristoyl, palmitoyl, stearoyl, linoleoyl, eicosanoyl, tricosanoyl and nonacosanoyl (derived from the fatty acids of the corresponding name: lauric, myristic, etc.). When given system names for the R_1 groups alone, the corresdonding names of the hydrocarbyl group derived from lauric acid is undecyl; from myristic acid, tridecyl; from palmitic acid, pentadecyl; from stearic



in which X_1 represents the residue of an acyl group after the rearrangement reaction as shown (from H to a complex organic group) while X_2 and X_3 independently represent H or an organic group. X_2 would initially represent R-CO-, but this group could be removed or be replaced by a different organic group using standard chemical reactions; since one of the two potential hydroxyl groups in the initial product is already protected, synthesis of compounds in which X_2 and X_3 represent different groups can readily be accomplished. Ions in which both X_2 and X_3 represent H are preferred, as these can be used in the synthesis of numerous imidazolinium compounds. Although there is no particular limit on the structure of the three "X" groups in the general synthesis other than those imposed by solubility or reactivity under the heating conditions being used for the reaction (which will be readily apparent), preferred organic groups are hydrocarbyl groups containing 30 or fewer carbons and their oxygenated products (especially fatty acids and their reaction products as previously described, as well as other hydrocarbyl groups and oxygenated products containing 15 or fewer carbon atoms, preferably 10 or fewer, more preferably hydrocarbyl groups containing no more than one phenyl ring with the remainder of the hydrocarbyl group being composed of alkyl groups, especially alkyl groups of 5 or fewer carbons). Organic groups formed from oxygenated hydrocarbyl groups are preferably carboxylic acids, alcohols, esters, ethers, ketones and aldehydes containing no more than one such functional group per organic group. Examples of imidazolinium ions that can be prepared by the synthesis as described above (with further modification of the hydroxyl groups using simple organic reactions) include 1,3-dihydroxyethylimidazolinium, 1-methoxyethyl-3-hydroxyethylimidazolinium, 1-

groups, as such "tri fatty acid" have been found not to be effective in transforming cells.

Because such techniques are generally known in the art, background information and basic techniques for the preparation of pharmaceutical compositions containing lipids will not be repeated at this time. A reader unfamiliar with this background information is referred to the publications under the heading Relevant Literature above and further to U.S. Patent No. 5,264,618. This last-cited patent describes a number of therapeutic formulations and methods in detail, including examples of the use of specific cationic lipids (different from those described here) that can be followed in detail by substituting the cationic lipids of the present invention for those described in the patent. Compositions of the present invention will minimally be useable in the manner described in the patent, although operating parameters may need to be modified in order to achieve optimum results, using the specific information provided for compounds of the invention in this specification along with the knowledge of a person skilled in the arts of lipid preparation and use.

The lipids of the present invention have been shown to be particularly useful and advantageous in the transfection of animal cells by genetic material. Additionally, since these compositions are degraded by enzymatic reactions in animal cells to components that are typically endogenous to the cells, the compositions provide a number of advantages in the area of low toxicity when compared to same previously known cationic lipids. These and other advantages of the invention are discussed in detail below. The remainder of this discussion is directed principally to selection, production, and use parameters for the cationic

are ligands or receptors capable of binding to some biological molecule of interest that is present in the target cell. A ligand can be any compound of interest which can specifically bind to another compound, referred to as a receptor, the ligand and receptor forming a complementary pair. The active compounds bound to the lipid mixture can vary widely, from small haptens (molecular weights of about 125 to 2,000) to antigens which will generally have molecular weights of at least about 6,000 and generally less than about 1 million, more usually less than about 300,000. Of particular interest are proteinaceous ligands and receptors that have specific complementary binding partners on cell surfaces. Illustrative active compounds include chorionic gonadotropin, encephalon, endorphin, luteinizing hormone, morphine, epinephrine, interferon, ACTH, and polyiodothyronines and fragments of such compounds that retain the ability to bind to the same cell-surface binding partners that bind the original (non-fragment) molecules.

The number of targeting molecules (either ligand or receptor) bound to a lipid mixture will vary with the size of the liposome, the size of the molecule, the binding affinity of the molecule to the target cell receptor or ligand, and the like. Usually, the bound active molecules will be present in the lipid mixture in from about 0.05 to 2 mole percent, more usually from about 0.01 to 1 mole percent based on the percent of bound molecules to the total number of molecules available in the mixture for binding.

The surface membrane proteins which bind to specific effector molecules (usually soluble molecules in the external environment of the cell) are referred to as receptors. In the present context, receptors include antibodies and immunoglobulins since these molecules are found on the surface of certain cells.

total lipid analyzed for the particular cationic lipid or its partial degradation product using, for example, HPLC.

The cationic amphiphiles are positively charged, and a tight charge complex can be formed between a cationic lipid carrier and a polyanionic nucleic acid, resulting in a lipid carrier-nucleic acid complex which can be used directly for systemic delivery to a mammal or mammalian cell. Where delivery is via aerosolization, the charge complex will withstand both the forces of nebulization and the environment within the lung airways and be capable of transfecting lung cells after the aerosolized DNA:lipid carrier complex has been deposited in the lung following intranasal or intraoral delivery of the aerosolized complex.

To evaluate the efficacy of a particular amphiphilic cation for use as a nucleic acid carrier in an aerosolization process, as well as to determine the optimum concentrations of lipid carrier-nucleic acid complexes, involves a two-step process. The first step is to identify lipid carriers and the concentration of lipid carrier-nucleic acid complexes that do not aggregate when the components are combined or during the significant agitation of the mixture that occurs during the nebulization step. The second step is to identify among those lipids that do not aggregate those complexes that provide for a high level of transfection and transcription of a gene of interest in target cells in the lung. These techniques are described in WO/US PCT/US92/11008 filed December 17, 1992, which disclosure is hereby incorporated by reference.

As an example, a reporter gene CAT (which encodes chloramphenicol acetyltransferase) can be inserted in an expression cassette and used to evaluate each lipid carrier composition of interest. The DNA:lipid carrier complexes are

for particular receptors may be employed, to target a cell associated with a particular surface protein. A particular ligand or antibody can be conjugated to the cationic amphiphile in accordance with conventional techniques, either by conjugating the site-directing molecule to a lipid for incorporation into the lipid bilayer or by providing a linking group on a lipid present in the bilayer for linking to a functionality of the site-directing compound. Such techniques are well known to those skilled in the art.

The various lipid carrier-nucleic acid complexes wherein the lipid carrier is a liposome are prepared using methods well known in the art. Mixing conditions can be optimized by visual examination of the resultant lipid-DNA mixture to establish that no precipitation occurs. To make the lipid-DNA complexes more visible, the complexes can be stained with a dye which does not itself cause aggregation, but which will stain either the DNA or the lipid. For example, Sudan black (which stains lipid) can be used as an aid to examine the lipid-DNA mixture to determine if aggregation has occurred. Particle size also can be studied with methods known in the art, including electron microscopy, laser light scattering, Coulter™ counting/sizing, and the like. Standard-size beads can be included as markers for determining the size of any liposomes or aggregates that form. By "lipid carrier-nucleic acid complex" is meant a nucleic acid sequence as described above, generally bound to the surface of a lipid carrier preparation, as discussed below. The lipid carrier preparation can also include other substances, such as enzymes necessary for integration, transcription and translation or cofactors. Furthermore, the lipid carrier-nucleic acid complex can include targeting agents to deliver the complex to particular cell or tissue types.

DNA to lipid carrier, minimizing the overall concentration of DNA:lipid carrier complex in solution, usually less than 5 mg DNA/8 ml solution, and avoiding the use of chelating agents such as EDTA and/or significant amounts of salt, either of which tends to promote macro-aggregation. The preferred excipient is water, dextrose/water or another solution having low or zero ionic strength. Further, the volume should be adjusted to the minimum necessary for deposition in the lungs of the host mammal, while at the same time taking care not to make the solution too concentrated so that aggregates form. Increasing the volume of the solution is to be avoided if possible due to the need to increase the inhalation time for the host animal to accommodate the increased volume. In some cases, it may be preferable to lyophilize the lipid carrier-nucleic acid complexes for inhalation. Such materials are prepared as complexes as described above, except that a cryoprotectant such as mannitol or trehalose is included in the buffer solution which is used for preparation of the lipid carrier-DNA complexes. Any glucose generally included in such a buffer is preferably omitted. The lipid carrier complex is rapidly freeze-dried following mixing of the lipid and DNA. The mixture can be reconstituted with sterile water to yield a composition which is ready for administration to a host animal.

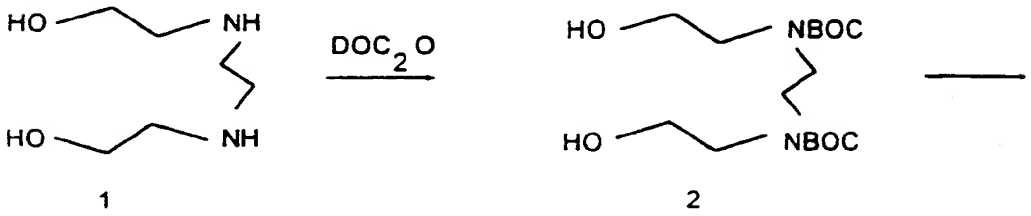
Where the amphiphiles form liposomes, the liposomes may be sized in accordance with conventional techniques, depending upon the desired size. In some instances, a large liposome injected into the bloodstream of an animal has higher affinity for lung cells as compared to liver cells. Therefore, the particular size range may be evaluated in accordance with any intended target tissue by administering lipid-nucleic acid complexes of varying particle sizes to a host

EXAMPLES

Example 1

Synthesis of 1-Acyloxyethyl-2-alkyl(alkenyl)-3-hydroxyethylimidazolinium derivatives

Commercially available N,N-bis(2-hydroxyethyl) ethylenediamine was N,N-diprotected using di-tert-butylpyrocarbonate, then O,O-diacylated using an appropriate acyl chloride. N-BOC protection groups were cleaved with 4M HCl in dioxane, and the resulting hydrochloride salt was subjected to thermal rearrangement in a suitable high boiling solvent to afford 1-acyloxyethyl-2-alkyl(alkenyl)-3-hydroxyethylimidazolinium derivatives:



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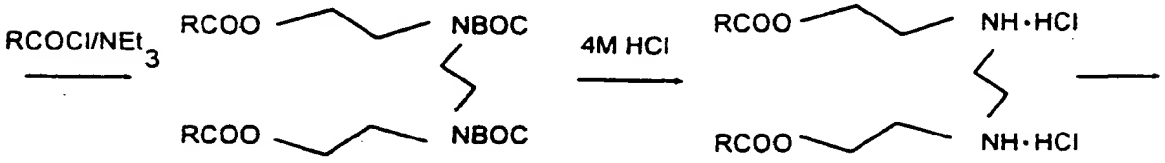
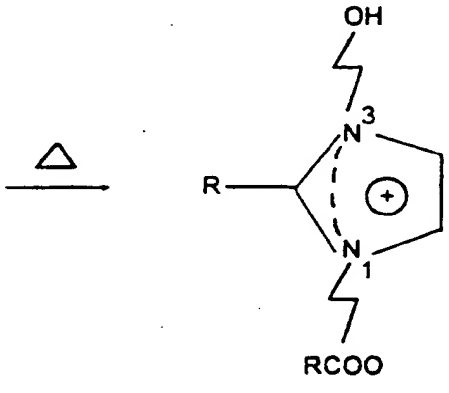


3 4



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solution of HCl in dioxane and the mixture was stirred at room temperature for 2 hrs. The resulting suspension was evaporated on rotavapor, diluted with ether (20 ml), filtered, washed with ether (15 ml x 2) and dried in vacuum to get 1.07g (100%) of diamino ester 4.

1-[9-(Z)-octadecenoyloxyethyl]-2-[8-(Z)-heptadecenyl]-3-hydroxyethylimidazolinium hydroxide (5.)

To a mixture of 1.16 g (0.00149 mol) of diamino ester 4 were added 3 ml of ethylene glycol and the mixture was stirred at 110°C (oil bath) for 30 min. The solution obtained was dissolved in 150 ml of $CHCl_3$ and washed with 5% NaCl (50 ml x 3) using MeOH. The organic layer was separated, dried over $MgSO_4$, evaporated on rotavapor and the rest was chromatographed on silica gel using 5-20% MeOH/$CHCl_3$ to get 0.95 g (75%) of product as yellowish oil.

(b) Transfection using liposomes containing 1-[9-(Z)octadecenoyloxyethyl]-2-[8-(Z)-heptadecenyl]-3-hydroxyethylimidazolinium compound 5.

Liposomes containing compound 5 in a 1:1 molar ratio with cholesterol was tested as a DNA carrier for gene transfer and expression in mice. The plasmid used was pZN51. The methods and plasmids used are described in more detail in WO93/24640. The liposomes were in a 10mM stock in 5% dextrose. The liposome:plasmid DNA ratios were screened for the presence of aggregation. Ratios from 1:2 to 1:7 (μg plasmid DNA to nanomoles cationic lipid) were screened. DNA:liposome ratios that did not produce aggregation were then tested in mice. 100 μg of pZN51 was complexed to 500 nanomoles of

without departing from the spirit or scope of the appended claims.

5. The amphiphile of claim 1 wherein R is pentadecyl or R_1 is hexadecanoyl.

6. The amphiphile of claim 1 wherein R is heptadecenyl or R_1 is octadecanoyl.

7. The amphiphile of claim 1 wherein said amphiphile is 1-[9-(Z)-octadecenoyloxyethyl]-2-[8-(Z)-heptadecenyl]-3-hydroxyethylimidazolinium.

8. A method of transforming cells, comprising:

contacting said cells with a plurality of complexes comprising an expression cassette and a nitrogen-containing amphiphile of Claim 1, wherein said complexes provide for transmission of cells in at least one tissue of said mammal and are susceptible to endogenous enzymatic cleavage to non-toxic products.

9. A method for transfecting a mammalian cell comprising contacting said cell with a complex comprising a transcription cassette or an expression cassette and a nitrogen-containing amphiphile of Claim 1.

10. A method for synthesizing an imidazolinium ion, which comprises:

heating a precursor compound of formula

INTERNATIONAL SEARCH REPORT

In tional application No.
PCT/US94/13363

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 25/26, 25/28; 43/04; A61K 31/70; C12N 15/00
US CL :424/417; 514/44; 435/172.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/417; 514/44; 435/172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts, CAS Online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,264,618 (FELGNER ET AL) 23 November 1993, col. 30, line 44 to col. 42, line 41.	9
X	Science, Volume 249, issued 14 September 1990, E.G. Nabel et al, "Site-Specific Gene Expression in Vivo by Direct Gene Transfer into the Arterial Wall", pages 1285-1288, see page 1286, fig. 2.	8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
14 FEBRUARY 1995	23 FEB 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Deborah Crouch. Ph.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

I. ational application No.
PCT/US94/13363

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-8, drawn to a nitrogen containing amphile and a method of transfecting cells of mammalian tissues, classified in Class 514, subclass 44 and Class 424, subclass 417.
Group II, claim 9, drawn to a method of transfecting mammalian isolated cells, classified in Class 435, subclass 172.3.
Group III, claim(s) 10-13, drawn to a method for synthesizing an imidazolinium ion, classified in Class 548, Subclass 335.1.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The in vivo and in vitro methods of groups I and II require separate and distinct protocols for transfection. In addition, neither method is required for the other method. The method of group III is unrelated to either the method of group I or group II in protocol. The method of group III is to make an organic compound. The methods of groups I and II are respectively to in vivo andinvitro transfections. The method of group III is not required for the method of groups I and II.